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Thyrotropic Activity of Various Adenohypophyseal Hormones of the Bullfrog

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ABSTRACT—The effects of adenohypophyseal hormones of the bullfrog (*Rana catesbeiana*) origin on the *in vitro* release of thyroxine (T_4) from the thyroid of prometamorphic larvae were studied. The bullfrog thyrotropin (TSH) preparation was 4 times as potent as bovine TSH in this model. Bullfrog luteinizing hormones (LH_S) (I-IV) and follicle-stimulating hormones (FSH_S) (I-IV), which were classified according to their isoelectric points, were tested for their thyrotropic activity and demonstrated about 10–40 and 1.5–3% of the activity of bullfrog TSH respectively. Similar activities were also found with LH and FSH preparations separated by immunoaffinity chromatography using monoclonal antibodies against the LH-IV β- and FSH-III β-subunits, respectively. Bullfrog prolactin, growth hormone and adrenocorticotropic hormone did not stimulate T_4 release from the thyroid gland into the medium. The results are discussed with reference to the known indirect (via the adenohypophysis) thyrotropic effects of mammalian thyrotropin-releasing hormone, corticotropin-releasing hormone and luteinizing hormone-releasing hormone.

INTRODUCTION

In amphibians, thyroid hormone has long been known to be the key hormone which induces metamorphic changes. However, the thyrotropic activity of amphibian adenohypophyseal hormones have not been determined fully, as all the amphibian thyrotropin (TSH) preparations used were contaminated significantly with luteinizing hormone (LH) or follicle-stimulating hormone (FSH) [1, 2] and other adenohypophyseal hormones, namely, prolactin (PRL), growth hormone (GH) and adrenocorticotropic hormone (ACTH), of amphibian origin have never been tested for their thyrotropic activity. Recently, we extracted a thyrotropic glycoprotein preparation, which is virtually free of LH and FSH, from the adult bullfrog pituitary gland [3]. The thyrotropic activities of this preparation and of other hormones (LH, FSH, PRL and GH) purified from the pituitary gland of the bullfrog (*Rana catesbeiana*) [4–7], were studied with a homologous bioassay system. The thyrotropic activity of bullfrog synthetic ACTH also was tested.

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MATERIALS AND METHODS

Hormones

Bovine (b) TSH was a gift from NIH (NIH-TSH-B5, 2.2 USP μ/mg). The bullfrog (f) TSH preparation was obtained from the adenohypophyses according to the method described previously [3], and fLHs (I-IV) and fESHs (I-IV), which have different isoelectric points (fLH-I, 8.8; II, 9.0; III, 9.1; IV, 9.3. fFSH-I 5.3; II, 5.7; III, 6.2; IV, 6.9) and gonadotropic activities [4, 5], were used. Other LH and FSH preparations, designated fLH-A and fFSH-A, respectively, obtained during the purification of fTSH by immunoaffinity chromatography [3] using monoclonal antibodies against the LH-IV β- and FSH-III β-snbunits [8, 9], respectively. Bullfrog PRL and

fGH were purified from adenohypophyses according to the methods of Yamamoto and Kikuyama [6] and of Kobayashi *et al.* [7] respectively. Bullfrog ACTH 1–39 was synthesizsed chemically by an automated synthesizer (Applied Biosystems Model 431A), according to the amino acid sequences of bullfrog α-melanophore-stimulating hormone (MSH) and corticotropin-like intermediate lobe peptide (CLIP) [10]. The adrenocorticotropic activity of the synthetic ACTH was assessed by measuring the release of aldosterone from the bullfrog adrenal tissue *in vitro* (Iwamuro *et al.*, unpublished).

Asssay for thyrotropic activity

Bullfrog tadpoles captured in a field were acclimatized to laboratory conditions at least for 1 week before use. A pair of thyroid glands with a small portion of the hyoid bone was excised from the lower jaw of stage XVIII and XIX larvae [11]. The explant was preincubated in 200 μl Dulbecco's modified Eagle's medium (GIBCO Laboratories), diluted to 66% with D. W., which contained 20 mM HEPES, 10 mM $NaHCO_3$, 20 mM glucose, 0.01% streptomycin sulfate, 100 IU/ml penicillin and 0.1% BSA (pH 7.5), for 1 hr at 22°C under 95% O_2-5% CO_2, after which the medium was renewed. Test substances were added and the incubation was continued for 12 or 24 hr. Finally, the medium was collected and thyroxine (T_4) concentrations were measured by radioimmunoassay (RIA). Comparison of thyrotropic activities of fTSH with other hormones was made using parallel line assay.

Radioimmunoassay for T_4

Thyroxine concentrations were measured by double antibody RIA. In this system, the cross reactivities of triiodothyronine (T_3), reverse T_3, monoiodotyrosine and diiodotyrosine were 0.66%, 0.56%, 0% and 0% respectively. The assay system consisted of 200 μl 1% BSA-phosphate buffered saline (PBS: 0.01 M sodium phosphate, 0.14 M NaCl and 0.01% merthiolate, pH 7.5), 100 μl standard hormone or sample, 100 μl antiserum diluted with 1% normal rabbit serum-0.05 M EDTA-PBS and 100 μl ^{125}I-labeled T_4 (Amersham) in 1% BSA-PBS, whichi was incubated overnight. After this incubation, 250 μl goat anti-rabbit γ-globulin serum diluted with 3.5% polyethylene glycol in 0.05 M EDTA-PBS was added to each tube, and incubated for 2 hr, after which the bound ^{125}I-labeled hormone was precipitated by centrifugation at 3,000 rpm for 15 min and the radioactivity was counted in a gamma counter. All these procedures were performed at room temperature.

Statistics. Statistical analysis was performed by analysis of variance.

RESULTS

Bovine TSH stimulated the *in vitro* release of T_4 from the thyroid of bullfrog larvae over a 24-hr period. After 12 hr incubation, the differences between 0.9 μg/ml and 0.3 μg/ml bTSH became evident. In the absence of bTSH, the T_4 levels in the medium remained extremely low (Fig. 1). Figure 2 shows the effects of various concentrations of bTSH in the medium on the release of T_4, which increased in proportion to the concentrations (0.1–0.9 μg/ml).

The response to fTSH was more marked than that to bTSH (Fig. 3). The dose-response curve of fTSH paralleled that of bTSH. The specific activ-

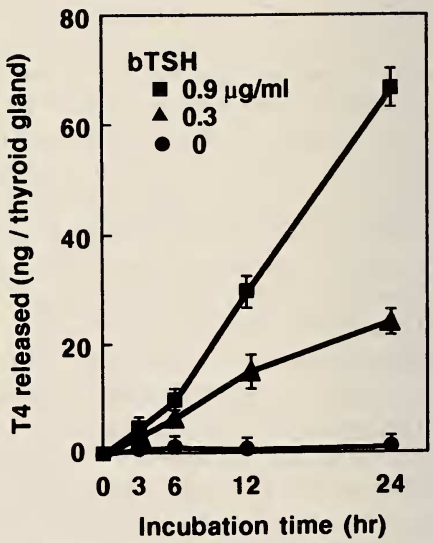

Fig. 1. Effect of bovine (b) TSH on thyroxine release from prometamorphic tadpole thyroid glands into the culture medium during a 24-hr incubation period. Values are means ± SEM (n=6).

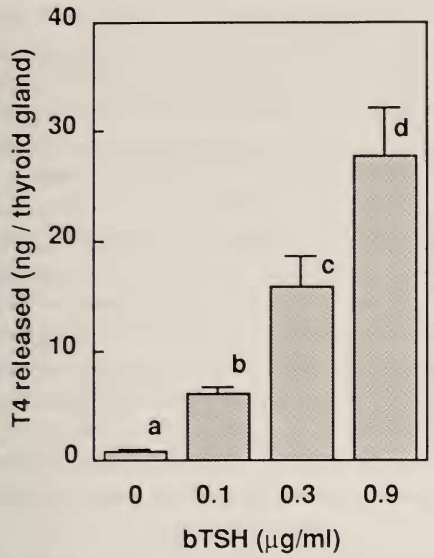

FIG. 2. Effects of various concentrations of bovine (b) TSH on thyroxine release from prometamorphic tadpole thyroid glands incubated for 12 hr *in vitro*. Values are means±SEM (n=6). Those with the same superscripts do not differ significantly at the 5% level.

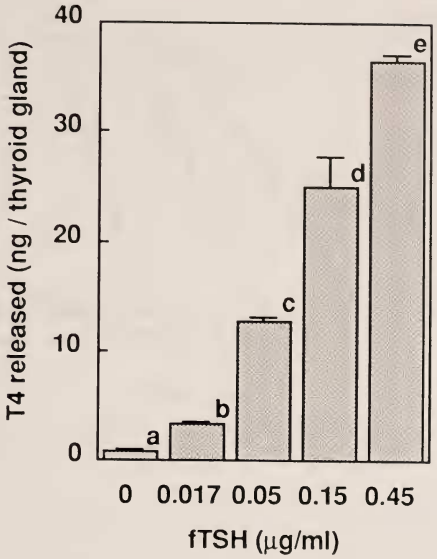

FIG. 3. Effects of various concentrations of bullfrog (f) TSH on thyroxine release from prometamorphic tadpole thyroid glands incubated for 12 hr. Values are means±SEM (n=6). Those with the same superscripts do not differ significantly at the 5% level.

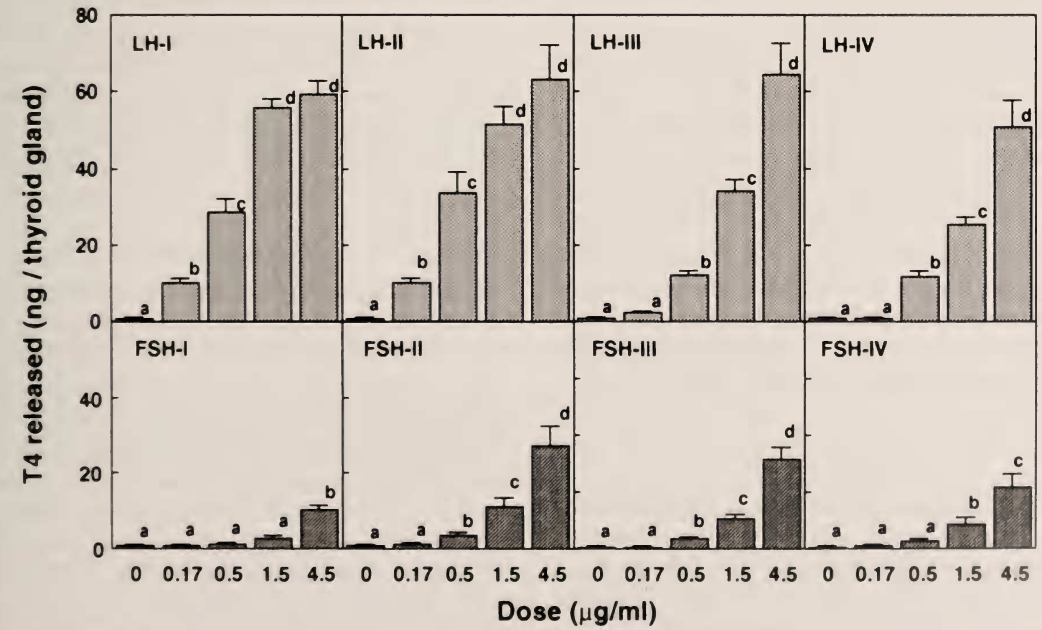

FIG. 4. Effects of various concentrations of bullfrog LHs (I-IV) and FSHs (I-IV) on thyroxine release from prometamorphic tadpole thyroid glands during a 12-hr incubation period. Values are means±SEM (n=6). Those with the same superscript do not differ significantly at the 5% level.

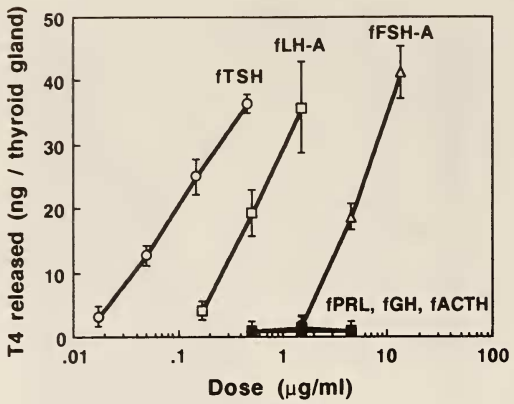

Fig. 5. Effects of various concentrations of bullfrog adenohypophyseal hormones (fLH-A, fFSH-A, fPRL, fGH and fACTH) on thyroxine release from prometamorphic tadpole thyroid glands during a 12-hr incubation period. LH and FSH were prepared by immunoaffinity chromatography. For comparison, the values for fTSH shown in Fig. 3. are also shown. Values are mean±SEM (n=6).

ity of fTSH was estimated to be about 4 times greater than that of bTSH.

The fLHs (I-IV) invariably exhibited thyrotropic activity and parallel line assay revealed that they possessed activities of 10–40% of that of fTSH (Fig. 4). The fFSHs (I-IV) were far less potent than the fLHs in stimulating T_4 release from the thyroid, retaining activities of 1.5–3% of that of fTSH (Fig. 4). The thyrotropic activities of fLH-A and fFSH-A, whihc were obtained during purification of fTSH by immunoaffinity chromatography using monoclonal antibodies against the fLH-IV β- and fFSH-III β-subunits, respectively, also were tested. The fLH-A exhibited thyrotropic activity of 20% of that of fTSH and that of fFSH-A wsa 2% (Fig. 5). Bullfrog PRL, fGH and fACTH did not stimulate the T_4 release (Fig. 5).

DISCUSSION

In this study, fTSH, which does not contain any measurable LH and FSH [3], exhibited the most potent thyrotropic activity of the bullfrog adenohypophyseal hormones tested. Interestingly, LH preparations exhibited a considerable thyrotropic activity. Purified gonadotropins are known to have an intrinsic ability to stimulate the thyroid glands of heterologous species [12]. According to MacKenzie and Licht [1], three anuran species, namely, *R. catesbeiana, R. pipiens* and *Hyla regilla,* showed a high degree of specificity for their bullfrog TSH preparation, whereas bullfrog LH exhibited a low thyrotropic activity. In contrast, bullfrog LH is more potent than the bullfrog TSH in stimulating the thyroid of the cockerel. In the present experiment, we employed a homologous bioassay system and, moreover, fLHs are regarded as highly purified ones, judging from their electrophoretic and chromatographic patterns and from the results of amino acid sequence analysis [5, 13]. In the case of LH obtained by immunoaffinity chromatography, immunoblotting analysis using antiserum against the human TSHβ subunit which recognizes fTSH [3] revealed the absence of TSH immunoreactivity in the LH preparation (Sakai *et al.*, unpublished). Therefore, the thyrotropic activity of fLHs observed in the present experiment seems to be derived from LHs themselves. With respect to the possession of thyrotropic activity by the fLHs, it is of interest to note that injections of mammalian luteinizing hormone-releasing hormone (LHRH) to ranid frogs increased plasma thyroxine levels [14] and that the medium in which a frog pituitery gland had been incubated in the presence of LHRH stimulated T_4 release from the thyroid *in vitro* [15]. In these cases, it is probable that LH, released in response to LHRH, rather than TSH, enhanced the T_4 release. It also should be mentioned that plasma T_4 levels in *Bufo japonicus* increase in the breeding season and are synchronized with the elevation of LH levels [16, 17].

Indirect effects of other hypothalamic releasing hormones on the thyroid has been studied both *in vitro* [15, 18] and *in vivo* [14, 19]. According to Denver and Licht [18], ovine corticotropin-releasing hormone (CRH) stimulated the release of thyrotropic factor(s) from the pituitary gland of the bullfrog and this effect is more marked in the larvae than in the adult. They also tested synthetic growth hormone-releasing hormone, but observed no indirect effect on the thyroid. In our study, neither ACTH nor GH enhanced T_4 release from the bullfrog thyroid. Accordingly, the indirect effect of CRH on the thyroid may not be mediated through ACTH. Although injections of mamma-

lian thyrotropin-releasing hormone (TRH) to tadpoles do not accelerate metamorphosis [20], mammalian TRH does appear to stimulate the release of thyrotropic factor(s) from the pituitary gland of adult amphibians [15, 18, 19].

The identity of TSH-releasing factor in amphibians has been sought for these past twenty years, but little progress has been made, as no specific and sensitive assay system for amphibian TSH has been developed and no attempt has been made to test the substances separated from the amphibian hypothalamus, instead of mammalian hypothalamic hormones, for their TSH-releasing effects on the amphibian pituitary. Most investigators evaluate the TSH-releasing activity of several mammalian hypothalamic hormones indirectly by measuring T_4 levels, rather than directly, by measuring TSH levels [14, 15, 18, 19]. Heretofore an amphibian TSH, which is pure enough for use in developing suitable radioimmunoassay system, has not been isolated. The TSH preparation used in this study has yet to be characterized fully, but it or a preparation purified further from it, appears promising for the future development of a radioimmunoassay for amphibian TSH.

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